PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/12, A61K 37/02
C12N 15/62, 15/13, C12P 21/02
C12N 5/10, C07K 13/00
C12P 21/08

(11) International Publication Number: WO 92/19735

A1

(43) International Publication Date: 12 November 1992 (12.11.92)

(21) International Application Number: PCT/US92/03755

(22) International Filing Date: 6 May 1992 (06.05.92)

(30) Priority data:
695,805 6 May 1991 (06.05.91) US
834,902 12 February 1992 (13.02.92) US

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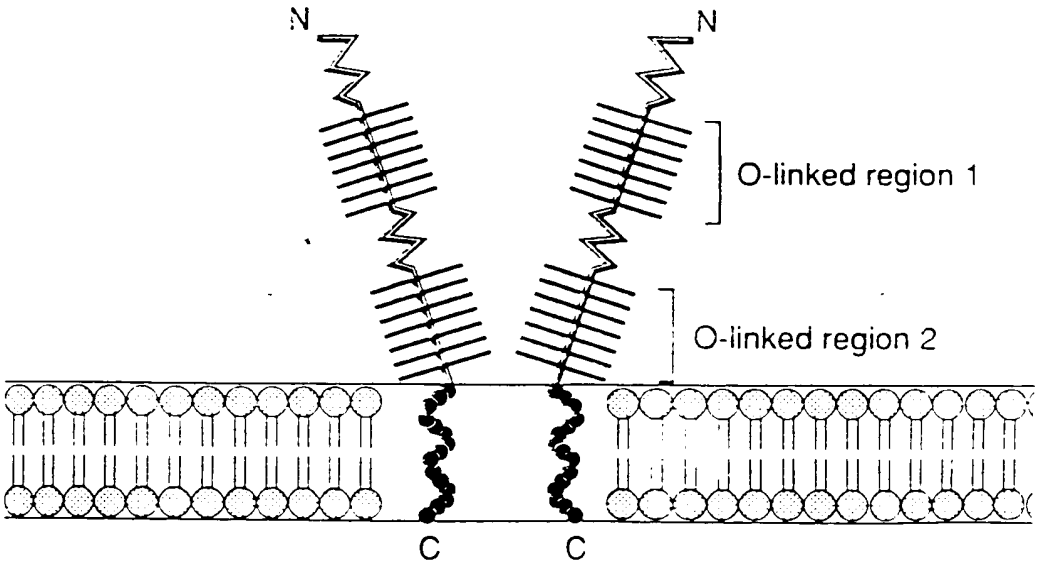
(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: GLYCAM-1 (Sgp 50), A SELECTIN LIGAND



(57) Abstract

The invention relates to glycoprotein ligands of selectins. The invention further relates to methods and means for preparing nd to nucleic acids encoding these ligands. The invention further concerns a method of treating a symptom or condition associated with excessive binding of circulating leukocytes to endothelial cells by administering to a patient in need of such treatment glycoprotein ligand of a selectin.

GLYCAM-1 (Sgp50), A SELECTIN LIGAND

BACKGROUND OF THE INVENTION

I. Field of the Invention

The present invention relates to endothelial selectin ligands. The invention further relates to methods and means for preparing and to nucleic acids encoding these ligands.

II. Description of Background and Related Art

Lymphocytes are mediators of normal tissue inflammation as well as pathologic tissue damage such as occurs in rheumatoid arthritis and other autoimmune diseases. In order to fully exploit the antigenic repertoire of the immune system, vertebrates have evolved a mechanism for distributing lymphocytes with diverse antigenic specificities to spatially distinct regions of the organism [Butcher, E.C., Curr. Top. Micro. Immunol. 128, 85 (1986); Gallatin *et al.*, Cell 44, 673 (1986); Woodruff *et al.*, Immunol. Today 10, 23 (1989); Yednock *et al.*, Adv. Immunol. 44, 313 (1989)].

This mechanism involves the continuous recirculation of the lymphocytes between the blood, where the cells have the greatest degree of mobility, and the lymphoid organs, where the lymphocytes encounter sequestered and processed antigen.

It has been recognized for some time that the trafficking of lymphocytes from the blood into secondary lymphoid organs, such as lymph nodes (LN) and gut-associated Peyer's patches (PP), is initiated by an adhesive interaction with the specialized endothelial cells of high endothelial venules (HEV) [Berg *et al.*, Immunol. Rev. 108, 5 (1989); Duijvestijn and Hamann, Immunol. Today 10, 23 (1989); Woodruff *et al.*, Annu. Rev. Immunol. 5, 201 (1987); Yednock and Rosen, Adv. Immunol. 44, 313 (1989); Stoolman, Cell 56, 907 (1989)]. Considerable evidence indicates that the lymphoid organ-selective migration or "homing" of lymphocytes is dictated in large part by organ-specific binding of lymphocytes to HEV [Butcher (1986), Supra]. Operationally, the lymphocyte-associated molecules underlying the organ-selective interaction with HEV are termed "homing receptors" while the cognate endothelial molecules are known as "HEV ligands" [Gallatin *et al.* (1986), Supra; Rosen, Curr. Opin. Cell. Biol. 1, 913 (1989)]. The endothelial HEV ligands are postulated to be distinctive for the different lymphoid organs and as such are proposed to be responsible for regulating the lymphocyte populations to enter each class of lymphoid organ [Butcher, Am. J. Pathol. 136, 3 (1990)]. A characterization of the detailed molecular mechanisms underlying lymphocyte trafficking is interesting from both a scientific and a clinical standpoint, since similar adhesive processes may be involved in both the normal and pathogenic forms of leukocyte inflammation [Watson *et al.*, Nature 349, 164-167 (1991)].

Of the homing receptors, the most thoroughly studied is a receptor initially termed peripheral lymph node homing receptor (pnHR). This receptor was first defined in the murine system by the MEL-14 monoclonal antibody (mAb), an antibody that was found to recognize

The three members of the LEC-CAM or selectin family of cell adhesion molecules are: L-selectin (a.k.a. peripheral lymph node homing receptor (pnHR), LEC-CAM-1, LAM-1, gp90^{MEL}, gp100^{MEL}, gp110^{MEL}, MEL-14 antigen, Leu-8 antigen, TQ-1 antigen, DREG antigen), E-selectin (LEC-CAM-2, LECAM-2, ELAM-1) and P-selectin (LEC-CAM-3, LECAM-3, GMP-140, PADGEM). These receptors will further on be referred to as "selectins". The structures of the selectin family members and of the genes encoding them are illustrated in Figures 1 and 2, respectively. The finding that simple monomeric sugars, such as mannose-6-phosphate (M6P) and fructose-1-phosphate, can block the interactions of murine and human lymphocytes with HEV of peripheral lymph nodes (pln) [Stoolman *et al.*, J. Cell Biol. 96, 722 (1983); Stoolman *et al.*, J. Cell Biol. 99, 1535 (1984); Stoolman *et al.*, Blood 70, 1842 (1987)] suggested that the endothelial ligand recognized by L-selectin is carbohydrate-based. In one series of experiments, Rosen and colleagues demonstrated that the homing receptor-dependent binding of lymphocytes to pln HEV was abolished by either *in vitro* or *in vivo* treatment with broad spectrum sialidases [Rosen *et al.*, Science (Wash., D.C.) 228, 1005 (1985); Rosen *et al.*, J. Immunol. 142, 1895 (1989)]. Because this enzyme selectively removes terminal sialic acid residues from oligosaccharides, these results strongly implied that sialic acid was a critical element for recognition.

The nature of the endothelial molecule(s) recognized by L-selectin was subsequently probed with a unique recombinant chimera, consisting of the extracellular d· ·ain of L-selectin joined to the hinge, CH2 and CH3 regions of the human IgG1 heavy chain [see WO 91/08298 published 13 June 1991 for the chimera, and Watson *et al.*, J. Cell Biol. 110, 2221 (1990) for its use as a probe for adhesive ligands of lymph node high endothelial venules]. Initial studies with this so-called receptor-immunoglobulin chimera demonstrated that it could adhere to (a) peripheral and mesenteric lymph node-specific HEV ligand(s) in cell blocking and immunohistochemical experiments [Watson *et al.* (1990), Supra]. The immunohistochemical recognition of this HEV ligand was abolished by treatment of lymph node sections with sialidase, suggesting that a component of the carbohydrate recognized by L-selectin was sialic acid-like and further accentuated the importance of the lectin domain in L-selectin-mediated adhesion [Rosen *et al.*, Science (Wash. D.C.) 228, 1005-1007 (1985); Rosen *et al.* (1989), Supra, and True *et al.*, J. Cell Biol. 11, 2757-2764 (1990)]. These results demonstrated the specificity of the L-selectin-immunoglobulin chimera for the pln HEV ligand and established that the ligand expresses carbohydrate residues that are essential for homing receptor-mediated cell adhesion.

A recent series of publications confirmed that the E-selectin ligand also has a carbohydrate character. Several laboratories, adopting a wide range of approaches, have concluded that an E-selectin ligand is a carbohydrate known as sialyl Lewis^x (sLex) or a closely related structure known as CD65 or VIM-2 [NeuAca2-3Galb1-4(Fuca1-3)GlcNAcb1]. Lowe *et al.*[Cell 63, 475 (1990)], transfected non-myeloid cells with an a1,3/4

These and further objects of the present invention will be apparent for one skilled in the art.

SUMMARY OF THE INVENTION

Our initial analysis of the HEV-associated ligand took advantage of a unique aspect of HEV metabolism. Early work by Andrews *et al.* [J. Cell Sci. 57, 277 (1982)] had shown that HEV in situ were distinctive in that they incorporated high amounts of inorganic sulfate into macromolecules. We have, therefore, analyzed the ability of L-selectin-IgG chimera to precipitate inorganic sulfate-labeled material from lymph nodes labeled with ^{35}S-sulfate in organ culture. A prominent 50 kD component and a weaker 90 kD molecule (Sgp^{50} and Sgp^{90}) were precipitated from lymph nodes but were not present in any other organ tested. The precipitation of these components with the L-selectin-IgG chimera was shown to be calcium-dependent, sensitive to both the MEL-14 mAb and specific carbohydrates. This reaction could be abolished by treatment of the sulfate-labeled proteins with sialidase or by inclusion of the carbohydrate polymer fucoidin in the reaction. Finally, a monoclonal antibody, termed MECA-79, which selectively reacts with so-called "vascular addressins" of pln HEV and blocks adhesivity for lymphocytes [Streeter *et al.*, J. Cell Biol. 107, 1853 (1988)], precipitated both components. A preliminary biochemical analysis revealed that the ~50 kD and ~90 kD L-selectin ligands were trypsin-sensitive glycoproteins, containing predominantly O-linked chains. [See Imai *et al.*, J. Cell Biol. 113, 1213 (1991).] The finding of O-linked chains is of interest in view of the evidence that O-linked regions cause cell surface glycoproteins to be highly extended and rigid structures [Jentoft *et al.*, Trends in Biochem Sci. 15, 291 (1990)] and thus ideally positioned to perform recognition functions. Fucose, sulfate and sialic acid were found in the O-linked chains of these molecules, and it is believed that fucose, like sialic acid, is required for full ligand activity.

In order to further characterize the nature of the endothelial ligand recognized by L-selectin, we have taken the novel approach of affinity purifying the sulfated ~50 kD HEV glycoprotein with the L-selectin-IgG chimera. The purified glycoprotein has been subjected to N-terminal amino acid sequencing, and this sequence information has been utilized to clone a cDNA encoding the protein component of this L-selectin ligand. It has been found that the cDNA encodes a novel, highly O-linked (mucin-like) glycoprotein that appears to function as a scaffold that presents carbohydrates to the lectin domain of L-selectin. Details of the experimental work along with the findings and their interpretation are provided in the examples.

The present invention concerns an isolated nucleic acid molecule comprising a nucleotide sequence encoding a selectin ligand.

In a still further aspect, the invention concerns a polypeptide as hereinbefore defined, further comprising an immunoglobulin constant domain sequence.

In a different aspect, the invention concerns a composition comprising an amount of a polypeptide (glycoprotein selectin ligand) as hereinabove defined, effective in blocking the binding of a corresponding selectin receptor to its native ligand, in admixture with a non-toxic, pharmaceutically acceptable excipient.

In another aspect, the invention relates to a method of treating a symptom or condition associated with excessive binding of circulating leukocytes to endothelial cells comprising administering to a patient in need of such treatment a polypeptide as hereinabove defined in an amount effective in blocking the binding of an L-selectin receptor on a circulating leukocyte to its endothelial ligand.

In still another aspect, the invention concerns an antibody immunoreactive with the protein part of a selectin ligand. Preferred antibodies bind the respective selectin ligand but will not substantially cross-react with any other known ligands, and will prevent the selectin ligands from binding to their receptors. The anti-selectin ligand antibodies may be immobilized, and in this form are, for example, useful for the detection or purification of the selectin ligands of the present invention.

In a further aspect, the invention relates to a method for determining the presence of a selectin ligand, comprising

a) hybridizing a nucleic acid encoding a selectin ligand or a complement of such nucleic acid to a test sample of nucleic acid; or

b) performing the polymerase chain reaction with primers based on a nucleic acid encoding a selectin ligand; and

c) determining the presence of a selectin ligand.

In a still further aspect, the invention provides a method for the purification of a selectin ligand comprising absorbing the ligand to a chimera comprising the corresponding selectin and an immunoglobulin heavy chain sequence.

The invention further concerns a method for presenting a selectin-binding moiety to a corresponding selectin by binding such moiety to the protein core of a selectin ligand glycoprotein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the structures of the selectin (LEC-CAM) family members as determined by cDNA cloning. Illustrated are the structures for L-selectin, E-selectin and P-selectin. The lectin, epidermal growth factor (EGF), and multiple short consensus repeats (SCRs) are shown with hypothetical disulfide bond structures as first proposed for GMP-140 by Johnston *et al.*, Cell 56, 1033 (1989). An N-terminal sequence is also shown (subsequently cleaved in the mature protein) as well as a hydrophobic transmembrane

P1 = Preimmune CAM01 - beads

P_S1 = Preimmune CAM01 - supernatent left after immunoprecipitation

I1 = Immune CAM01 - beads

I_S1 = Immune CAM01 - supernatent left after immunoprecipitation

P_S2 = Preimmune CAM02 - supernatent left after immunoprecipitation

I_S2 = Immune CAM02 - supernatent left after immunoprecipitation

I2 = Immune CAM02 - beads

PEP = free peptide

Figure 6. Northern blot analysis of the expression of the mRNA encoding the ~50 kD L-selectin ligand. A. Total(a) or poly A+ (b, c) RNA was isolated from normal (a,b) or inflamed (c) peripheral lymph nodes, run on formaldehyde gels and analyzed by Northern blot analysis with the cDNA shown in Figure 4. B. Poly A+ RNA was isolated from a) brachial, b) axillary, c) cervical, d) popliteal, and e) total peripheral lymph node and hybridized on Northern blots with the ligand cDNA as described in A., C. and D. Poly A+ RNA was isolated from a) peripheral lymph nodes, b) liver, c) Peyer's patch, d) thymus, e) skeletal muscle, f) mesenteric lymph nodes, g) testes, h) lung, i) heart, j) spleen, k) brain, and l) kidney and hybridized on Northern blots with C. the cDNA corresponding to the L-selectin ligand or D. a chicken beta actin cDNA.

Figure 7. In Situ hybridization analysis of the expression of the mRNA encoding the ~50 kD L-selectin ligand. Peripheral lymph node sections were hybridized with either an anti-sense (A) or sense (B) hybridization probe corresponding to the L-selectin ligand cDNA, washed, exposed to emulsion for 6 weeks and developed. The morphology of the HEV is shown with a dotted line surrounding the venule.

Figure 8. A model of the structure of the ~50 kD Selectin ligand. Illustrated is one possible model for the structure of the ~50 kD L-selectin ligand on the luminal surface of the peripheral lymph node HEV. The extended brush-like regions correspond to O-linked regions I and II in a highly O-glycosylated state. The less-extended regions correspond to the N-terminal and central serine/threonine poor domains. In this model, membrane attachment is accomplished by oligomerization of the C-terminal amphipathic helical regions and insertion of these regions into the membranes so that the polar regions interact with each other to form an oligomer and the apolar faces of the helices interact with the lipid bilayer. As described in the text, a number of other models are also equally likely.

DETAILED DESCRIPTION OF THE INVENTION

I. DEFINITIONS

The term "selectin ligand" and its grammatical variants, are used to refer to a polypeptide having a qualitative biological property in common with a naturally occurring ligand of a selectin molecule.

a half-life greater than about 20 hours. Examples of suitable stable plasma proteins are immunoglobulins, albumin, lipoproteins, apolipoproteins and transferrin. The amino acid sequence having a qualitative biological property in common with a naturally occurring selectin ligand is generally fused C-terminally to a stable plasma protein sequence, e.g. immunoglobulin constant domain sequence.

The term "immunoglobulin" generally refers to polypeptides comprising a light or heavy chain usually both disulfide bonded in the native "Y" configuration, although other linkage between them, including tetramers or aggregates thereof, is within the scope hereof.

Immunoglobulins (Ig) and certain variants thereof are known and many have been prepared in recombinant cell culture. For example, see U.S. Patent 4,745,055; EP 256,654; Faulkner *et al.*, Nature 298:286 (1982); EP 120,694; EP 125,023; Morrison, J. Immun. 123:793 (1979); Köhler *et al.*, Proc. Nat'l. Acad. Sci. USA 77:2197 (1980); Raso *et al.*, Cancer Res. 41:2073 (1981); Morrison *et al.*, Ann. Rev. Immunol. 2:239 (1984); Morrison, Science 229:1202 (1985); Morrison *et al.*, Proc. Nat'l. Acad. Sci. USA 81:6851 (1984); EP 255,694; EP 266,663; and WO 88/03559. Reassorted immunoglobulin chains also are known. See for example U.S. patent 4,444,878; WO 88/03565; and EP 68,763 and references cited therein. Ligand binding protein-stable plasma protein chimeras, and specifically L-selectin-immunoglobulin chimeras are, for example, disclosed in WO 91/08298 published 13 June 1991. The immunoglobulin moiety in the chimera of the present invention may be obtained from IgG_1, IgG_2, IgG_3, or IgG_4 subtypes, IgA, IgE, IgD or IgM, but preferably IgG_1 or IgG_3.

Selectin, such as L-selectin binding can, for example, be assayed by determining the binding of radiolabeled (e.g. ^{35}S-labeled) ligands to immobilized receptor-immunoglobulin chimera, in the presence or absence of soluble inhibitors, essentially as described by Imai *et al.*, J. Cell Biol. 113, 1213 (1991). Alternatively or in addition, adherence to cells expressing the respective receptor can be used to assay ligand binding. For example, EL-4 cells (ATCC TIB39) are known to express high levels of L-selectin on their surfaces, and can therefore be used in cell adhesion assays for L-selectin ligands. Adherent cells can be quantitated by lactate dehydrogenase activity (Bradley *et al.*, J. Cell. Biol. 105, 991 (1987)).

The terms "nucleic acid molecule encoding", "DNA sequence encoding", and "DNA encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide chain. The DNA sequence thus codes for the amino acid sequence.

The term "isolated" when used in relation to a nucleic acid or a protein refers to a nucleic acid or protein that is identified and separated from at least one containment nucleic acid or protein with which it is ordinarily associated in its natural source. Isolated nucleic acid or protein is such present in a form or setting that is different from that in which it is found

The introduced DNA sequence may be from the same species as the host cell or a different species from the host cell, or it may be a hybrid DNA sequence, containing some foreign and some homologous DNA.

"Ligation" refers to a process of forming phosphodiester bonds between two nucleic acid fragments. To ligate the DNA fragments together, their ends must be compatible. In some cases, the ends will be directly compatible after endonuclease digestion. However, it may be necessary to first convert the staggered ends commonly produced after endonuclease digestion to blunt ends to make them compatible for ligation. To blunt ends, the DNA is treated in a suitable buffer for at least 15 minutes at 15°C with about 10 units of the Klenow fragment of DNA polymerase I or T4 DNA polymerase in the presence of the four deoxyribonucleotide triphosphates. The DNA is then purified by phenol-chloroform extraction and ethanol precipitation. The DNA fragments that are to be ligated together are put in solution in about equimolar amounts. The solution will also contain ATP, ligase buffer, and a ligase such as T4 DNA ligase at about 10 units per 0.5 μg of DNA. If the DNA is to be ligated into a vector, the vector is first linearized by digestion with the appropriate restriction endonuclease(s). The linearized fragment is then treated with bacterial alkaline phosphatase, or calf intestinal phosphatase to prevent self-ligation during the ligation step.

The terms "amino acid" and "amino acids" refer to all naturally occurring L-α-amino acids. This definition is meant to include norleucine, ornithine, and homocysteine. The amino acids are identified by either the single-letter or three-letter designations:

Asp	D	aspartic acid	Ile	I	isoleucine
Thr	T	threonine	Leu	L	leucine
Ser	S	serine	Tyr	Y	tyrosine
Glu	E	glutamic acid	Phe	F	phenylalanine
Pro	P	proline	His	H	histidine
Gly	G	glycine	Lys	K	lysine
Ala	A	alanine	Arg	R	arginine
Cys	C	cysteine	Trp	W	tryptophan
Val	V	valine	Gln	Q	glutamine
Met	M	methionine	Asn	N	asparagine

These amino acids may be classified according to the chemical composition and properties of their side chains. They are broadly classified into two groups, charged and uncharged. Each of these groups is divided into subgroups to classify the amino acids more accurately:

I. Charged Amino Acids

Acidic Residues: aspartic acid, glutamic acid

Basic Residues: lysine, arginine, histidine

Deletional variants are those with one or more amino acids in the native selectin ligand amino acid sequence removed. Ordinarily, deletional variants will have one or two amino acids deleted in a particular region of the molecule.

An essential role of the protein core of the present selectin ligands is to present the specific carbohydrate structure recognized by a selectin receptor to the respective receptor.

Accordingly, any alteration within the two highly O-glycosylated, serine- and threonine-rich regions (amino acids 42-63 and amino acids 93-122 in Figure 4) of the L-selectin ligand amino acid sequence is expected to have more significant effect on the lymphocyte-high endothelial venule interaction than changes in other regions of the protein. As it will be shown hereinbelow, the highly O-glycosylated regions are essential to provide a rigid, inflexible "bottle brush" structure that allows for the large number of O-linked carbohydrate ligands attached to the serine and threonine residues to be appropriately presented to the leukocyte surface-localized L-selectin lectin domains, thereby mediating the carbohydrate-dependent adhesive interaction. Alterations within these regions are expected to result in molecules the receptor binding activities of which will be significantly different from that of the corresponding native ligand.

The glycoprotein ligands of the present invention comprise fucose, sialic acid and an anionic component, preferably sulfate esters as O-linked carbohydrate components, and it is believed that fucose, like sialic acid, and sulfate are required for full ligand activity.

Examples of specific carbohydrate components of the glycoprotein ligands of the invention can be expressed as follows:

NeuNAcα2-3Galβ1-4(Fucα1-3)GlcNAc

NeuNAcA2-3Galβ1-4GlcNAcB1-3GalB1-4(FucA1-4(FucA1-3)GlcNAc.

"Northern blot analysis" is a method used to identify RNA sequences that hybridize to a known probe such as an oligonucleotides, DNA fragment, cDNA or fragment thereof, or RNA fragment. The probe is labeled with a radioisotope such as ^{32}P, or by biotinylation, or with an enzyme. The RNA to be analyzed is usually electrophoretically separated on an agarose or polyacrylamide gel, transferred to nitrocellulose, nylon, or other suitable membrane, and hybridized with the probe, using standard techniques well known in the art such as those described in sections 7.39-7.52 of Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, New York: Cold Spring Harbor Laboratory Press, 1989.

"Oligonucleotides" are short-length, single- or double-stranded polydeoxynucleotides that are chemically synthesized by known methods (such as phosphotriester, phosphite, or phosphoramidite chemistry, using solid phase techniques such as those described in EP 266,032, published 4 May 1988, or via deoxynucleoside H-phosphanate intermediates as described by Froehler *et al.*, Nucl. Acids Res. 14, 5399 (1986)). They are then purified on polyacrylamide gels.

chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (e.g., Fab, $F(ab')_2$, and Fv). Cabilly, et al., U.S. Pat. No. 4,816,567; Mage & Lamoyi, in Monoclonal Antibody Production Techniques and Applications, pp.79-97 (Marcel Dekker, Inc., New York, 1987).

Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from such a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method.

II. GENERAL METHODS

A. Obtaining DNA Encoding A Selectin Ligand

The DNA encoding a selectin ligand may be obtained from any cDNA library prepared from tissue believed to possess mRNA for the selectin ligand and to express it at a detectable level. An L-selectin ligand gene thus may be obtained from a cDNA library prepared from (mesenteric or peripheral) lymph nodes. Genes encoding the other selectin ligands can be prepared from other cDNA libraries in an analogous manner.

Libraries are screened with probes designed to identify the gene of interest or the protein encoded by it. For cDNA expression libraries, suitable probes usually include mono- and polyclonal antibodies that recognize and specifically bind to the desired protein; oligonucleotides of about 20-80 bases in length that encode known or suspected portions of the selectin ligand cDNA from the same or different species; and/or complementary or homologous cDNAs or their fragments that encode the same or similar gene.

An alternative means to isolate the gene encoding a selectin ligand, e.g. an L-selectin ligand, is to use polymerase chain reaction (PCR) methodology as described in section 14 of Sambrook *et al.*, Supra or in Chapter 15 of Current Protocols in Molecular Biology, Supra.

Another alternative is to chemically synthesize the gene encoding the desired selectin ligand using one of the methods described in Engels *et al.*, Agnew. Chem. Int. Ed. Engl. 28, 716 (1989). These methods include triester, phosphite, phosphoramidite and H-Phosphonate methods, PCR and other autoprimer methods, and oligonucleotide syntheses on solid supports. These methods may be used if the entire nucleic acid sequence of the gene is known, or the sequence of the nucleic acid complementary to the coding strand is available, or, alternatively, if the target amino acid sequence is known, one may infer potential nucleic acid sequences, using known and preferred coding residues for each amino acid residue.

A preferred method for practicing this invention is to use carefully selected oligonucleotide sequences to screen cDNA libraries from various tissues, preferably mammalian lymph node high endothelial venules (L-selectin ligand), or myeloid cells (E-selectin and P-selectin ligands). Among the preferred mammals are humans and members of the following orders: bovine, ovine, equine, murine, and rodentia.

site(s), an oligonucleotide is ligated into the site where the foreign DNA has been cut. The oligonucleotide is designed to code for the desired amino acids to be inserted and additionally has 5' and 3' ends that are compatible with the ends of the foreign DNA that have been digested, such that direct ligation is possible.

2. Oligonucleotide-Mediated Mutagenesis

Oligonucleotide-directed mutagenesis is the preferred method for preparing the substitution variants of this invention. It may also be used to conveniently prepare the deletion and insertion variants of this invention. This technique is well known in the art as described by Adelman et al. (DNA, 2:183 [1983]).

Generally, oligonucleotides of at least 25 nucleotides in length are used to insert, delete or substitute two or more nucleotides in the t-PA molecule. An optimal oligonucleotide will have 12 to 15 perfectly matched nucleotides on either side of the nucleotides coding for the mutation. This ensures that the oligonucleotide will hybridize properly to the single-stranded DNA template molecule. The oligonucleotides are readily synthesized using techniques well known in the art such as that described by Crea et al. (Proc. Nat'l. Acad. Sci. USA, 75:5765 [1978]).

The DNA template molecule is the single-stranded form of the vector with its wild-type cDNA t-PA insert. The single-stranded template can only be generated by those vectors that are either derived from bacteriophage M13 vectors (the commercially available M13mp18 and M13mp19 vectors are suitable), or those vectors that contain a single-stranded phage origin of replication as described by Veira et al. (Meth. Enzymol., 153:3 [1987]). Thus, the cDNA t-PA that is to be mutated must be inserted into one of these vectors in order to generate single-stranded template. Production of the single-stranded template is described in sections 4.21-4.41 of Sambrook et al., Supra.

To mutagenize the native selectin ligand sequence, the oligonucleotide is annealed to the single-stranded DNA template molecule under suitable hybridization conditions. A DNA polymerizing enzyme, usually the Klenow fragment of E. coli DNA polymerase I, is then added. This enzyme uses the oligonucleotide as a primer to complete the synthesis of the mutation-bearing strand of DNA. Thus, a heteroduplex molecule is formed such that one strand of DNA encodes the native selectin ligand inserted in the vector, and the second strand of DNA encodes the mutated form of the selectin ligand inserted into the same vector. This heteroduplex molecule is then transformed into a suitable host cell, usually a prokaryote such as E. coli JM101. After growing the cells, they are plated on to agarose plates and screened using the oligonucleotide primer radiolabeled with 32-P to identify the colonies that contain the selectin ligand mutated in its protein core. These colonies are selected, and the DNA is sequenced to confirm the presence of mutations in the protein core of the molecule.

Mutants with more than one amino acid substituted may be generated in one of several ways. If the amino acids are located close together in the polypeptide chain, they may be

standard DNA technology. Mutations at separate positions can be introduced simultaneously by either using a mutant second primer or performing a second PCR with different mutant primers and ligating the two resulting PCR fragments simultaneously to the vector fragment in a three (or more)-part ligation.

C. Insertion Of DNA Into A Replicable Vector

The cDNA or genomic DNA encoding the (native or variant) selectin ligands of the present invention is inserted into a replicable vector for further cloning or expression. Many vectors are available, and selection of the appropriate vector will depend on 1) whether it is to be used for DNA amplification (cloning) or for expression, 2) the size of the DNA to be inserted into the vector, and 3) the host cell to be transformed with the vector. Each vector contains various components depending on its function and the host cell with which it is compatible. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter and a transcription terminator sequence. Specific vectors will be discussed hereinbelow in conjunction with the host cells with which they are compatible.

Suitable vectors are prepared using standard recombinant DNA procedures. Isolated plasmids and DNA fragments are cleaved, tailored, and ligated together in a specific order to generate the desired vectors.

The DNA is cleaved using the appropriate restriction enzyme or enzymes in a suitable buffer. In general, about 0.2-1 μg of plasmid or DNA fragments is used with about 1-2 units of the appropriate restriction enzyme in about 20 μl of buffer solution. (Appropriate buffers, DNA concentrations, and incubation times and temperatures are specified by the manufacturers of the restriction enzymes.) Generally, incubation times of about one or two hours at 37°C are adequate, although several enzymes require higher temperatures. After incubation, the enzymes and other contaminants are removed by extraction of the digestion solution with a mixture of phenol and chloroform, and the DNA is recovered from the aqueous fraction by precipitation with ethanol.

To ligate the DNA fragments together to form a functional vector, the ends of the DNA fragments must be compatible with each other. In some cases the ends will be directly compatible after endonuclease digestion. However, it may be necessary to first convert the sticky ends, commonly produced by endonuclease digestion, to blunt ends to make them compatible for ligation. To blunt the ends, the DNA is treated in a suitable buffer for at least 15 minutes at 15°C with 10 units of the Klenow fragment of DNA Polymerase I (Klenow) in the presence of the four deoxynucleotide triphosphates. It is then purified by phenol-chloroform extraction and ethanol precipitation.

The cleaved DNA fragments may be size-separated and selected using DNA gel electrophoresis. The DNA may be electrophoresed through either an agarose or a

1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor cells (MMT 060562, ATCC CCL 51); rat hepatoma cells (HTC, MI.54, Baumann et al., J. Cell Biol., 85:1 [1980]); and TRI cells (Mather et al., Annals N.Y. Acad. Sci., 383:44 [1982]). Expression vectors for these cells ordinarily include (if necessary) DNA sequences for an origin of replication, a promoter located in front of the gene to be expressed, a ribosome binding site, an RNA splice site, a polyadenylation site, and a transcription terminator site.

Promoters used in mammalian expression vectors are often of viral origin. These viral promoters are commonly derived from polyoma virus, Adenovirus2, and most frequently Simian Virus 40 (SV40). The SV40 virus contains two promoters that are termed the early and late promoters. These promoters are particularly useful because they are both easily obtained from the virus as one DNA fragment that also contains the viral origin of replication (Fiers et al., Nature, 273:113 [1978]). Smaller or larger SV40 DNA fragments may also used, provided they contain the approximately 250-bp sequence extending from the HindIII site toward the BglI site located in the viral origin of replication.

Alternatively, promoters that are naturally associated with the foreign gene (homologous promoters) may be used provided that they are compatible with the host cell line selected for transformation.

An origin of replication may be obtained from an exogenous source, such as SV40 or other virus (e.g., Polyoma, Adeno, VSV, BPV) and inserted into the cloning vector. Alternatively, the origin of replication may be provided by the host cell chromosomal replication mechanism. If the vector containing the foreign gene is integrated into the host cell chromosome, the latter is often sufficient.

Satisfactory amounts of selectin ligand can be produced by transformed cell cultures. However, the use of a secondary DNA coding sequence can enhance production levels. The secondary coding sequence typically comprises the enzyme dihydrofolate reductase (DHFR). The wild-type form of DHFR is normally inhibited by the chemical methotrexate (MTX). The level of DHFR expression in a cell will vary depending on the amount of MTX added to the cultured host cells. An additional feature of DHFR that makes it particularly useful as a secondary sequence is that it can be used as a selection marker to identify transformed cells.

Two forms of DHFR are available for use as secondary sequences, wild-type DHFR and MTX-resistant DHFR. The type of DHFR used in a particular host cell depends on whether the host cell is DHFR deficient (such that it either produces very low levels of DHFR endogenously, or it does not produce functional DHFR at all). DHFR-deficient cell lines such as the CHO cell line described by Urlaub and Chasin (Proc. Natl. Acad. Sci. (USA) 77:4216 [1980]) are transformed with wild-type DHFR coding sequences. After transformation, these

26:205 (1983); Yelton et al., Proc. Natl. Acad. Sci. USA, 81:1470 (1984)] and A. niger [Kelly and Hynes, EMBO J., 4:475 (1985)].

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzeman et al., J. Biol. Chem., 255:2073 [1980]) or other glycolytic enzymes (Hess et al., J. Adv. Enzyme Reg., 7:149 [1968]; Holland et al., Biochemistry, 17:4900[1978]), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In the construction of suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination. Other promoters that have the additional advantage of transcription controlled by growth conditions are the promoter region for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Any plasmid vector containing yeast-compatible promoter, origin of replication and termination sequences is suitable.

3. Prokaryotic Cells

Prokaryotes are particularly useful for rapid production of large amounts of DNA, for production of single-stranded DNA templates used for site-directed mutagenesis, for screening many mutants simultaneously, and for DNA sequencing of the mutants generated. Suitable prokaryotic host cells include E. coli K12 strain 294 (ATCC number 31,446), E. coli strain W3110 (ATCC number 27,325) E. coli X1776 (ATCC number 31,537), and E. coli B; however many other strains of E. coli, such as HB101, JM101, NM522, NM538, NM539, and many other species and genera of prokaryotes may be used as well.

Prokaryotes may also be used as hosts for expression of DNA sequences. The E. coli strains listed above, bacilli such as Bacillus subtilis, other enterobacteriaceae such as Salmonella typhimurium or Serratia marcesans, and various Pseudomonas species may all be used as hosts.

Plasmid vectors containing replicon and control sequences that are derived from species compatible with the host cell are used with these hosts. The vector usually has a replication site, marker genes that provide phenotypic selection in transformed cells, one or more promoters, and a polylinker region containing several restriction sites for insertion of foreign DNA. Plasmids typically used for transformation of E. coli include pBR322, pUC18, pUC19, pUC118, pUC119, and Bluescript M13, all of which are described in sections 1.12-1.20 of Sambrook et al., Supra. However, many other suitable vectors are available as well. These vectors contain genes coding for ampicillin and/or tetracycline resistance which enables cells transformed with these vectors to grow in the presence of these antibiotics.

New York (1988), p. 769) and can be used as signal sequences in appropriate eukaryotic host cells. Yeast signal sequences, as for example acid phosphatase (Arima et al., Nuc. Acids Res., 11:1657 (1983)), alpha-factor, alkaline phosphatase and invertase may be used to direct secretion from yeast host cells. Prokaryotic signal sequences from genes encoding, for example, LamB or OmpF (Wong et al., Gene 68:193 1988)), MalE, PhoA, or beta-lactamase, as well as other genes, may be used to target proteins from prokaryotic cells into the culture medium.

An alternative technique to provide a protein of interest with a signal sequence such that it may be secreted is to chemically synthesize the DNA encoding the signal sequence. In this method, both strands of an oligonucleotide encoding the selected signal sequence are chemically synthesized and then annealed to each other to form a duplex. The double-stranded oligonucleotide is then ligated to the 5' end of the DNA encoding the protein.

The construct containing the DNA encoding the protein with the signal sequence ligated to it can then be ligated into a suitable expression vector. This expression vector is transformed into an appropriate host cell and the protein of interest is expressed and secreted.

E. Transformation Methods

Cultures of mammalian host cells and other host cells that do not have rigid cell membrane barriers are usually transformed using the calcium phosphate method as originally described by Graham and Van der Eb (Virology, 52:546 (1978)) and modified as described in sections 16.32-16.37 of Sambrook et al. Supra. However, other methods for introducing DNA into cells such as Polybrene (Kawai and Nishizawa, Mol. Cell. Biol., 4:1172 (1984)), protoplast fusion (Schaffner, Proc. Natl. Acad. Sci. USA, 77:2163 (1980)), electroporation (Neumann et al., EMBO J., 1:841 (1982)), and direct microinjection into nuclei (Capecchi, Cell, 22:479 (1980)) may also be used.

Yeast host cells are generally transformed using the polyethylene glycol method, as described by Hinnen (Proc. Natl. Acad. Sci. U.S.A., 75:1929 (1978)).

F. Culturing The Host Cells

The mammalian host cells used to produce the selectin ligands of the present invention may be cultured in a variety of media. Commercially available media, such as Ham's F10 (Sigma), Minimal Essential Medium (MEM, Sigma), RPMI-1640 (sigma), or Dulbecco's Modified Eagle's Medium (DMEM, Sigma) are suitable for culturing such host cells. In addition, any of the media described in Ham and Wallace, Meth. Enz. 58, 44 (1979); Barnes and Sato, Anal. Biochem. 102, 255 (1980); U.S. Patent Nos. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; U.S. Patent Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin and/or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and

(1987). Glycosylation is suppressed by tunicamycin as described by Duskin *et al.*, J. Biol. Chem. 25?, 3105 (1982). Tunicamycin blocks the formation of protein-N-glycosydase linkages.

Glycosylation variants of the selectin ligands herein can also be produced by selecting appropriate host cells. Yeast, for example, introduce glycosylation which varies significantly from that of mammalian systems. Similarly, mammalian cells having a different species (e.g. hamster, murine, insect, porcine, bovine or ovine) or tissue (e.g. lung, liver, lymphoid, mesenchymal or epidermal) origin than the source of the selectin ligand, are routinely screened for the ability to introduce variant glycosylation as characterized for example, by elevated levels of mannose or variant ratios of mannose, fucose, sialic acid, and other sugars essential for selectin binding.

H. Covalent Modifications

Covalent modifications of a naturally occurring selectin ligand molecule or a sequence having a biological property in common with such molecule, are included within the scope herein. Such modifications are traditionally introduced by reacting targeted amino acid residues of the selectin ligand protein with an organic derivatizing agent that is capable of reacting with selected side-chains or terminal residues, or by harnessing mechanisms of post-translational modifications that function in selected recombinant host cells. The resultant covalent derivatives are useful in programs directed at identifying residues important for biological activity, for immunoassays of the selectin ligands, or for the preparation of anti-selectin ligand antibodies for immunoaffinity purification of the recombinant glycoprotein. For example, complete inactivation of the biological activity of the protein after reaction with ninhydrin would suggest that at least one arginyl or lysyl residue is critical for its activity, whereafter the individual residues which were modified under the conditions selected are identified by isolation of a peptide fragment containing the modified amino acid residue. Such modifications are within the ordinary skill in the art and are performed without undue experimentation.

Derivatization with bifunctional agents is useful for preparing intramolecular aggregates of the selectin ligand glycoprotein with polypeptides as well as for cross-linking the selectin ligand glycoprotein to a water insoluble support matrix or surface for use in assays or affinity purification. In addition, a study of interchain cross-links will provide direct information on conformational structure. Commonly used cross-linking agents include 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, homobifunctional imidoesters, and bifunctional naleimides. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable intermediates which are capable of forming cross-links in the presence of light. Alternatively, reactive water insoluble matrices such as cyanogen bromide activated carbohydrates and the systems reactive substrates described in U.S. patent Nos. 3,959,642; 3,969,287; 3,691,016; 4,195,128; 4,247,642;

[Kobet *et al.*, Supra], or analogous sites of other immunoglobulins) is used in the fusion. In a particularly preferred embodiment, the amino acid sequence containing the selectin binding site(s) is fused to the hinge region and C_H2 and C_H3 or C_H1, hinge, C_H2 and C_H3 domains of an IgG_1, IgG_2 or IgG_3 heavy chain. The precise site at which the fusion is made is not critical, and the optimal site can be determined by routine experimentation.

J. Purification Of The Selectin Ligands

The selectin ligand may be recovered and purified from recombinant cell cultures by known methods, including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, hydroxyapatite chromatography, immunoaffinity chromatography and lectin chromatography. Other known purification methods within the scope of this invention utilize reverse-phase HPLC chromatography using anti-selectin ligand antibodies are useful for the purification of the ligands of the present invention.

A particularly advantageous purification scheme, specifically developed for the purification of the L-selectin ligand, will be described in Example 1. This method takes advantage of a unique selectin receptor-immunoglobulin chimera (referred to as L-selectin-IgG), produced by recombinant methods, which is able to precipitate the corresponding (sulfate-labeled) ligand.

K. Therapeutic Compositions

The selectin ligands of the present invention can be used to block the binding of a corresponding selectin receptor to its native ligand. For example, the L-selectin ligand effectively blocks the binding of an L-selectin receptor on a circulating leukocyte to its native ligand on an endothelial cell. This property is useful for treating a symptom or condition associated with excessive binding of circulating leukocytes to endothelial cells, such as inflammation associated with rheumatoid arthritis, psoriasis, multiple sclerosis, etc.

The selectin ligands of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby the ligand is combined in admixture with a pharmaceutically acceptable carrier. Suitable carriers and their formulations are described in Remington's Pharmaceutical Sciences, 16th ed., 1980, Mack Publishing Co., edited by Oslo et al. These compositions will typically contain an effective amount of the ligand, for example, from on the order of about 0.5 to about 10 mg/ml, together with a suitable amount of carrier to prepare pharmaceutically acceptable compositions suitable for effective administration to the patient. The ligands may be administered parenterally or by other methods that ensure its delivery to the bloodstream in an effective form.

Compositions particularly well suited for the clinical administration of the ligands used to practice this invention include sterile aqueous solutions or sterile hydratable powders such as lyophilized protein. Typically, an appropriate amount of a pharmaceutically acceptable salt is also used in the formulation to render the formulation isotonic.

by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The affinity of the monoclonal antibody for binding the corresponding ligand can, for example, be determined by the Scatchard analysis of Munson & Pollard, Anal. Biochem. 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods. Goding, Monoclonal Antibodies: Principles and Practice, pp.59-104 (Academic Press, 1986). Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies of the invention is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences, Morrison, et al., Proc. Nat. Acad. Sci. 81:6851 (1984), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. In that manner, "chimeric" or "hybrid" antibodies are prepared that have the binding specificity of an anti-selectin ligand monoclonal antibody herein.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody of the invention, or they are substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for a selectin ligand and another antigen-combining site having specificity for a different antigen.

Chimeric or hybrid antibodies also may be prepared in vitro using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a

III. EXAMPLES

EXAMPLE 1

Identification of Surface Glycoproteins on Endothelial Cells Recognized by L-selectin

This example shows that recombinant L-selectin selectively binds $^{35}SO_4$-labeled macromolecules from lymph nodes. In particular, two sulfated, fucosylated and sialylated glycoproteins have been identified.

A. Metabolic Labeling of Organs with ^{35}S-sulfate

Mesenteric or peripheral (cervical, brachial, axillary) lymph nodes were collected from 8-16 week old female ICR mice. The lymph nodes were cut into 1 mm thick slices with a razor blade and the slices (typically, 0.2 g of wet weight) were suspended in 1 ml of RPMI-1640 containing 25 mM HEPES, 100 U/ml Penicillin G, 100 μg/ml streptomycin, and 200 μCi carrier-free [^{35}S] sodium sulfate (ICN Biochemicals Inc., Costa Mesa, CA) according to the procedure of Ager, J. Cell Sci., 87:133 (1987). After incubation of 37°C for 4 hr, the slices were washed extensively in Dulbecco's phosphate-bufferred saline (PBS), and then homogenized in 1 ml of lysis buffer (2% Triton X-100 in PBS containing 1 mM PMSF, 1% (v/v) aprotinin, 10 μg/ml pepstatin, 0.02% NaN_3) with a Potter-Elvehjem homogenizer on ice. Lysis was contained for 1 hr on a rocker at 4°C. The lysate was centrifuged at 10,000 x g for 1 hr at 4°C. EDTA was added to the supernatant at a final concentration of 2 mM and the supernatant was precleared by rocking with Affi-Gel Protein A (250 μl of packed beads, BioRad Laboratories, Richmond, CA) overnight at 4°C.

B. Identification of the Components Adsorbed to L-selectin-IgG Beads

Affi-Gel Protein A (10 μl packed beads) was incubated with 30 μg of either L-selectin-IgG (WO 91/08298 published 13 June 1991), CD4-IgG (prepared according to Capon et al., Nature 337:525 (1989) or human IgG_1 (Calbiochem, La Jolla, CA) in 1 ml of PBS rocking overnight at 4°C. The beads (referred to as L-selectin-IgG beads, CD4-IgG beads and hulgG-beads) were washed 3X in PBS and once with lysis buffer. The CD4-IgG and hulgG beads were used as controls.

The precleared lysate described in Section A, above, was centrifuged at 10,000 x g for 10 sec, $CaCl_2$ was added to the supernatant at a final concentration of 5 mM, and the supernatant was mixed immediately with either L-selectin-IgG beads, CD4-IgG beads or hulgG-beads (typically 200 μl of precleared lysate per 10 μl packed beads), and incubated for 4 hr at 4°C on a rocker. The beads were washed 6X with lysis buffer, transferred to a new tube and washed once more with lysis buffer.

The materials bound to the L-selectin-IgG beads were solubilized by boiling in SDS in the presence of 2-mercaptoethanol, electrophoresed on SDS-polyacrylamide gels (9 or 10%) and subjected to fluorography with ENTENSIFY or EN^{3}HANCE (NEN). By fluorography, the 50 D component tended to be more diffuse with ENTENSIFY than EN^{3}HANCE. In the

EXAMPLE 2

Purification of the 50 kD L-selectin ligand for cloning and sequence determination

The work described in Example 1 demonstrated that the L-selectin-IgG chimera could be utilized to biochemically characterize the ~50 kD sulfated endothelial ligand produced by peripheral and mesenteric lymph nodes. Further work has demonstrated that this ligand is readily shed into the medium when peripheral lymph nodes (PLN) are placed into organ culture (S. Watson-unpublished observations). Thus, the initial step in the purification of the L-selectin ligand for sequence determination was to produce large quantities of medium conditioned by murine PLN. A second observation that allowed for a dramatic purification was that ~50 kD sulfated L-selectin ligand was soluble after treatment of conditioned medium with chloroform-methanol. This step resulted in a >350 fold purification of the sulfated ligand. The next purification step consisted of a wheat germ agglutinin affinity column, which took advantage of the apparently high content of carbohydrate in this ligand. The final purification step utilized an L-selectin-IgG chimera affinity column to purify the ligand. This final step assured that the material contained within the ~50 kD region would correspond to a glycoprotein that could bind with relatively high affinity to L-Selectin.

Mesenteric or peripheral (cervical, brachial, and axillary) lymph nodes were collected from 8-16-wk-old female ICR mice. Mice were killed and their mesenteric lymph nodes were removed. Typically, a single batch of conditioned medium was made from the mesenteric nodes of 30 mice. Occasionally, a small number (approximately 5% of the total lymph node weight) of peripheral lymph nodes were also added. The nodes were cut into approximately 1-mm-thick slices with a razor blade and the slices were added to the standard cell culture medium RPMI-1640 supplemented with 25 mM HEPES buffer, 1 U/ml penicillin and 1 μg/ml streptomycin in a 100-ml cell culture bottle. The ratio of medium to nodes was 6 ml/30 mesenteric lymph nodes.

The culture bottle was placed in a 37°C incubator. After 4 hours, the medium was poured into a 15-ml conical tube and centrifuged at 500 x g for 10 minutes to remove large tissue debris. The supernatant was centrifuged again in a 15-ml Corex tube at 20,000 x g for 15 minutes. The resultant supernatant was first poured through Nitex screen to remove fatty particles that do not pellet during centrifugation, and was then snap-frozen with liquid nitrogen and stored at -20°C.

For the purpose of monitoring the protein purification scheme, $^{35}SO_4$-labeled Sgp50 was added to the conditioned medium prepared as hereinabove described. This material was prepared by labeling 5 mice mesenteric lymph nodes in 1 ml of the above-described culture medium with 0.5 mCi $Na^{35}SO_4$ (ICN). After 4 hours, the conditioned cell culture medium was removed and centrifuged in a microfuge for 10 minutes. The supernatant was removed and precleared by adding to 100 μl packed protein A-agarose beads (Zymed Corp.), and rocking

Biosystems Inc.), stained with Coomassie R-250 and destained. The blot was air-dried and exposed to Kodak XAR film to detect the position of the sulfate labelled ligand. This region of the gel was cut out and subjected to gas-phase microsequencing. Sequencing was essentially performed as hereinbefore described.

Polypeptide sequencing revealed an unambiguous stretch of 25 amino acids at approximately the 5 pM level (Figure 3B).

EXAMPLE 4

cDNA Cloning and Sequence Analysis of the ~50 kD L-Selectin Ligand

A murine peripheral lymph node cDNA library was constructed using an InvitroGen cDNA library kit and poly A + RNA isolated from murine peripheral lymph nodes. A redundant oligonucleotide probe pool was derived from residues 9-17 of the N-terminal sequence (QMKTQPMDA) using degenerate codons selected on the basis of the mammalian codon usage rule. Codons were CAG, ATG, AAG, AAA, ACA, ACT, ACC, CCA, CCT, CCC, GAT, or GAC. Only GC was used for the 5' Ala codon. The 26-mer oligonucleotide was ^{32}P labeled by polynucleotide kinase and hybridized to duplicate nitrocellulose filters derived from 20 plates containing 1 million gT10 bacteriophage in 20% formamide, 5X SSC (150 mM CaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH7.6), 5X Denhardt's solution, 10% dextran sulfate and 20 micrograms per ml denatured, sheared salmon sperm DNA at 42°C overnight. The filters were washed in 1X SSC, 0.1% SDS at 42°C twice for 30 minutes and autoradiographed at -70°C overnight. A single duplicate positive phage was plaque purified, and the Eco R1 insert was subcloned into the pGEM vector. The entire nucleotide sequence of both strands was obtained by supercoin sequencing with the Sequenase kit. For in situ hybridization and Northern blot analysis, a polymerase chain reaction fragment lacking the poly A tail was synthesized then subcloned into the pGEM vector (PROMEGA). The nucleotide sequence of the encoded cDNA is shown in Figure 4. The clone contained a short (about 600 bp) cDNA with a single open reading frame of 151 amino acids. A "Kozak box" (CCACCATGA) was found surrounding the first encoded methionine [Kozak, M. Cell Biology 115:887 (1991)]. This methionine was followed by a 19 amino acids long, highly hydrophobic sequence that appears to function as a signal sequence for translocation of the protein into the secretory pathway. This region is followed by a sequence corresponding almost exactly to that determined by N-terminal sequencing of the L-selectin-IgG bound material. The signal sequence-processed 132 amino acid protein is extremely rich in serine and threonine, with about 29% of the encoded amino acids corresponding to these residues.

Perhaps more interestingly, these serine and threonine residues were found to be clustered in two regions of the glycoprotein (Figure 3D). Region I (residues 42-63) was found to contain 12 serine or threonine residues (~55%) while region II (residues 93-122) was

sulfate labeled L-selectin ligand that was purified by binding to the L-selectin-IgG chimera as described above.

To verify that the cloned protein is the same as the ^{35}S-labeled material purified from conditioned medium with the L-selectin IgG chimera, immunoprecipitation of L-selectin-IgG purified ^{35}S-labeled material was performed. The following procedure was used for two separate experiments. For the preparation of immunoprecipitation beads, 25 µl packed protein A-Sepharose beads (Zymed Laboratories) + 25 µl rabbit serum + 350 µl PBS are rocked together in a microfuge tube for 3 hours, 4°C. Each tube is washed 3 times with PBS to remove unbound immunoglobulin and only the 25 µl beads remains. 60 µl of PBS containing approximately 6,000 cpm of L-selectin-IgG purified ^{35}S-labeled material is added. This is incubated on ice for 3 hours, flicking the tube every 15 minutes. After 3 hours, the microfuge tube is spun to pellet the beads. 45 µl of supernatant is taken off and mixed with 15 µl 4X Laemmli sample buffer and boiled for SDS-PAGE analysis. The pelleted beads are washed 3 times with PBS, transferred to a new tube, and supernatant decanted to leave 45 µl final volume in tube. 15 µl 4X Laemmli sample buffer is added and the tubes are boiled for SDS-PAGE analysis. This SDS-gel run was under reducing conditions. Immunoglobulin heavy chain runs at 50 kD under reducing conditions and the labeled band is compressed. In this experiment, none of the preimmune sera interact with the label, whereas, CAM01 and CAM05 have partial effects and CAM02 totally immunoprecipitates the band. This experiment was repeated with CAM02 with the following differences. The gel was run under non-reducing conditions so that the 50 kD band would not be compressed. (We have previously established that the L-selectin-IgG purified ^{35}S-labeled material does not change mobility in an SDS-gel under reducing conditions.) Also, for one tube, the CAM02 antibody coated-beads were preincubated with 1 mg/ml CAM02 peptide for 30 minutes on ice in order to show specificity of the antibody-antigen interaction. Finally, an irrelevant control peptide antibody against the C-terminus peptide of L-selectin (called ROSY 1B), also prepared by Caltag using similar protocols, was tested. Both gels were subjected to fluorography with Enhance (New England Nuclear) and autoradiography with Kodak Xar film. CAM02 completely immunoprecipitates the L-selectin-IgG purified ^{35}S-labeled material, CAM02 preimmune and ROSY 1B have no effect. The free CAM02 peptide blocks the specific immunoprecipitation. The results are shown in Figures 5A and B.

EXAMPLE 6

Expression of the L-Selectin Ligand

Figure 6 shows a Northern blot analysis of the mRNA encoding the ~50 kD L-selectin ligand. As can be seen in Figure 6A, the mRNA is encoded in the poly A+ fraction and corresponds to a discrete band of ~0.7 kD. The sharpness of the band argues against a significant level of alternative RNA splicing, and rescreening of the murine PLN cDNA library with the isolated ligand clone has not revealed any other spliced forms of the message.

strand encoded by the isolated ligand cDNA clone clearly hybridizes to the HEV of peripheral lymphoid tissue, while the sense strand shows no significant hybridization. This result clearly demonstrates that the mRNA corresponding to the ligand cDNA is synthesized by HEV cells, consistent with previous immunohistochemical data demonstrating the localization of the L-selectin ligand to this region of the mesenteric and PLN.

The data described here are consistent with the hypothesis that an endothelial ligand for L-selectin is a unique mucin-type glycoprotein. Mucins, by definition, are serine/threonine rich proteins whose molecular weight is predominantly due to O-linked carbohydrate side chains (Cyster et al., The Embo J. 10:893 (1991), Fukuda, M., Glycobiology 1:347 (1991), Gendler et al., Am. Rev. Respir. Dis. 144:S42 (1991), Gum et al., The J. of Biol. Chem. 266:22733 (1991), Porchet et al., Am. Rev. Resp. Dis. 144:S15 (1991)). The high serine and threonine content found in the L-selectin ligand described here, coupled with the high degree of olycosylation of the protein (~70% by molecular weight), suggests that the bulk of the carbohydrates on the ligand are, in fact, O-linked and confirms previous experiments demonstrating N-glycanase resistance of the ~50 kD sulfated PLN ligand. The fact that the O-linked carbohydrates appear to be directly involved in the adhesive interactions mediated by the L-Selectin lectin domain suggests that the role of the protein backbone described here appears to be as a scaffold for carbohydrate presentation. This protein, therefore, represents a novel type of cell adhesion molecule that functions to present carbohydrates in a tissue-specific manner to the lectin domain of L-selectin. In this way, the regional expression of this "scaffold" may result in regional trafficking of lymphocyte populations.

The use of a mucin-like glycoprotein as a scaffold for carbohydrate presentation to a selectin makes sense when viewed in the context of what is currently known about mucin structure. Previous investigations into the structures of highly O-linked glycoproteins such as mucins have revealed that these molecules tend to be highly extended, somewhat rod-like molecules. For example, the leukocyte surface mucin leukosialin (sialophorin, CD43) (Cyster et al. 1991, Supra, Fukuda 1991, Supra), has been demonstrated to form a rigid, rod-like structure, and physico-chemical analyses of other mucins have demonstrated similar rod-like conformations, particularly in the highly O-glycosylated regions (Harding, S.E., Advances in Carbohydrate Chemistry and Biochemistry 47:345 Academic Press, Inc. (1989), Jentoft, N., TIBS 15:291 (1990)). In addition, other non-mucin proteins, such as decay accelerating factor (DAF) and the low density lipoprotein (LDL) receptor contain highly O-linked domains near the cell surface that appear to form rod-like domains that may function to extend the receptors through the glycocalyx (Jentoft 1990, supra). This rod-like structure is exactly what would be expected of a molecule whose role is to present carbohydrates to the lectin domain of a selectin. As shown in the model illustrated in Figure 6, the L-selectin ligand may be thought of as a "bottle brush" that extends into the lumen of the HEV. This would allow for a large number of O-linked carbohydrate ligands (the bristles on the brush) to be

The rapid shedding of the ligand reported here suggests a relatively loose association with the luminal surface of the HEV. One such association could be mediated by the amphipathic helix described above and illustrated in the model shown in Figure 8. This helical region could span the membrane and simultaneously mediate membrane attachment and the formation of oligomeric forms of the ligand. That the ligand is capable of oligomerization has been found during gel filtration experiments (Y. Imai and S. Rosen-unpublished observations). A number of other proteins have been found to utilize amphipathic helices for membrane association and pore formation (Haffar et al., J. Cell Biol. 107:1677 [1988], Eisenberg et al., J. Mol. Biol. 179:125 [1984], Finer-Moore and Stroud, Proc. Natl. Acad. Sci. USA 81:155 [1984]), and it is therefore possible that this domain could function in a similar manner in the case of the L-selectin ligand. An alternative hypothesis is that the amphipathic helix could interact weakly with another protein that is more tightly associated with the endothelial cell surface. It is also possible that the ligand is incorporated into the glycocalyx in a currently ill-defined manner. A final possibility is that there are several HEV ligands that bind to the L-selectin lectin domain, some of which are tightly associated with the endothelial cell surface, such as the ~90 kD sulfated ligand described by Imai et al (1991), Supra or the PLN addressins described by Streeter et al., J. Cell Biol. 107, 1853 (1988b), and others, like the ~50 kD ligand described here, that are shed.

The relationship between the mucin-like endothelial ligand described here and the previously reported group of proteins defined by the monoclonal antibody MECA 79 (the pln "addressins" Streeter et al., Nature (Lond.) 331:41, J. Cell Biol. 107, 1853 [1988], Berg et al., Immunol. Rev. 108:5 [1991]) remains to be defined. Imai et al. (1991), Supra previously demonstrated that the ligand described here is recognized by the MECA 79 antibody (an antibody that binds an unknown carbohydrate determinant), but Streeter et al. (1988b), Supra and Berg et al. (1991), Supra have shown that a number of additional glycoproteins appear to also express this carbohydrate-like epitope. It is, therefore, possible that other endothelial glycoproteins exist that present carbohydrate to the L-selectin lectin domain. The development of monoclonal antibody reagents specific for the mucin-like ligand reported here will therefore be of great importance, since they will allow for an assessment of the relative contribution of this glycoprotein versus others as adhesive ligands for L-selectin-mediated trafficking.

The ~50 kD L-selectin ligand is the fourth type of molecule that is involved with cell adhesion in the immune system: 1) the leukocyte integrins, 2) their ligands, the immunoglobulin (Ig) superfamily members, 3) the selectins and 4) the ~50 kD L-selectin ligand. The integrins, Ig superfamily members, and selectins have all been found to comprise families containing a diversity of related molecules. Because of the characteristics of the ligand described here, we propose to replace the cumbersome nomenclature used throughout

CLAIMS:

1. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a selectin ligand.

2. The nucleic acid molecule of claim 1 comprising a nucleotide sequence encoding a glycoprotein having a carbohydrate structure recognized by a selectin receptor.

3. The nucleic acid molecule of claim 1 comprising a nucleotide sequence able to hybridize to the complement of a nucleotide sequence encoding a protein having the amino acid sequence shown in Figure 4.

4. The nucleic acid molecule of claim 1 comprising a nucleotide sequence encoding a selectin ligand protein having an amino acid sequence greater than about 40% homologous with the amino acid sequence shown in Figure 4.

5. The nucleic acid molecule of claim 1, selected from the group consisting of:

(a) a cDNA clone having a nucleotide sequence derived from the coding region of a native selectin ligand gene;

(b) a DNA sequence able to hybridize under low stringency conditions to a clone of (a); and

(c) a genetic variant of any of the DNA sequences of (a) and (b) which encodes a glycoprotein possessing a biological property of a naturally occurring ligand of a selectin molecule.

6. The nucleic acid molecule of claim 1 comprising a nucleotide sequence encoding an L-selectin ligand.

7. The nucleic acid molecule of claim 6 comprising the coding region of the nucleotide sequence shown in Figure 4, or a genetic variant thereof which encodes a glycoprotein possessing a biological property of a naturally occurring ligand of the L- selectin molecule.

8. The nucleic acid molecule of claim 1 further comprising a nucleotide sequence encoding an immunoglobulin constant domain.

9. The nucleic acid molecule of claim 8 wherein the immunoglobulin is IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgD or IgM.

10. The nucleic acid molecule of claim 7 further comprising a promoter operably linked to the coding region of the nucleotide sequence shown in Figure 4.

11. An expression vehicle comprising a nucleotide sequence encoding an L-selectin ligand operably linked to control sequences recognized by a host cell transformed with the vehicle.

12. A host cell transformed with the expression vehicle of claim 11.

13. The host cell of claim 12 which is an eukaryotic cell.

14. The host cell of claim 13 which is a mammalian cell.

such treatment a polypeptide of claim 18 in an amount effective in blocking the binding of an L-selectin receptor on a circulating leukocyte to its endothelial ligand.

32. The method of claim 31 wherein said glycoprotein is administered as a pharmaceutical formulation comprising a non-toxic, pharmaceutically acceptable excipient.

33. The method of claim 32 further comprising the administration of an antiinflammatory or an antineoplastic drug.

34. An antibody immunoreactive with the protein part of a selectin ligand.

35. The antibody of claim 34 wherein the ligand is L-selectin ligand.

36. A method for determining the presence of a selectin ligand, comprising

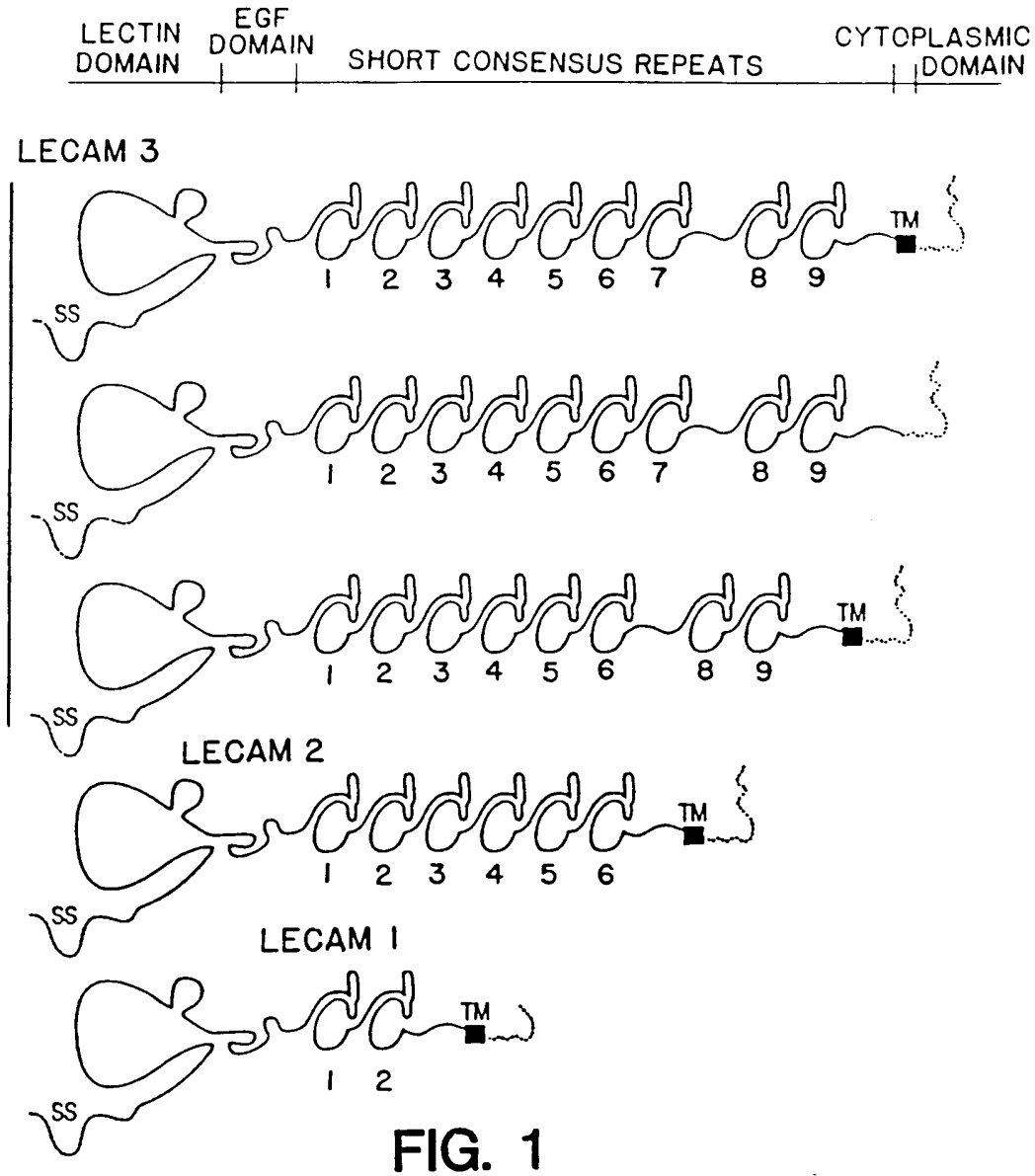
a) hybridizing a nucleic acid encoding a selectin ligand or a complement of such nucleic acid to a test sample of nucleic acid; or

b) performing the polymerase chain reaction with primers based on a nucleic acid encoding a selectin ligand; and

c) determining the presence of a selectin ligand.

37. A method for the purification of a selectin ligand comprising the use of a chimera comprising the corresponding selectin and an immunoglobulin heavy chain sequence.

38. A method for presenting a selectin-binding moiety to a corresponding selectin comprising binding said moiety to the polypeptide of claim 19.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EGF DOMAIN
SHORT CONSENSUS REPEATS
CYTOPLASMIC DOMAIN
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SS
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TM
SS
1 2 3 4 5 6 7 8 9
TM
SS
1 2 3 4 5 6 8 9
TM
LECAM 2
SS
1 2 3 4 5 6
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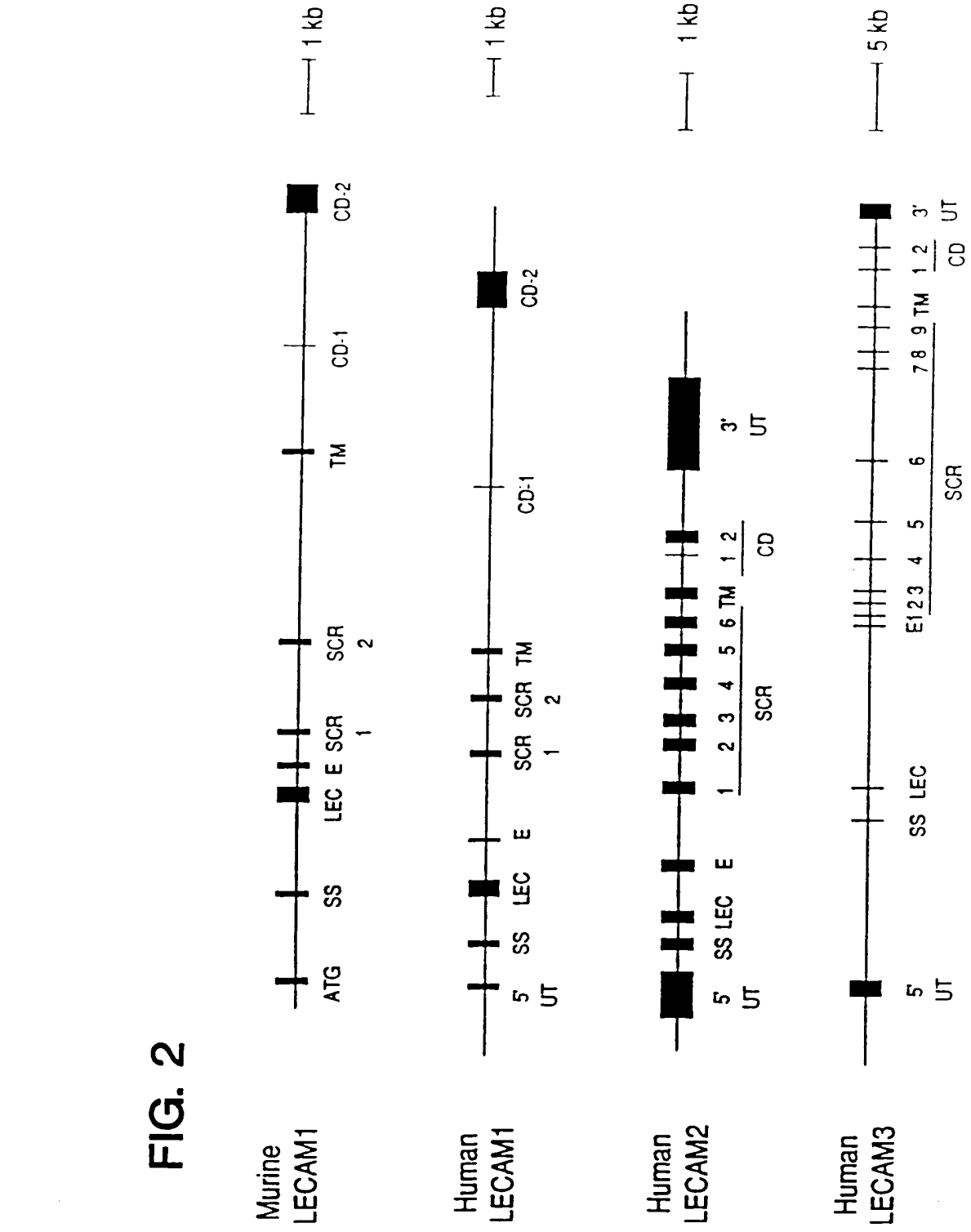
LECAM 1
SS
1 2
TM

FIG. 1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SS
LEC
E
SCR
1
SCR
2
TM
CD-1
CD-2
Murine LECAM1
1 kb
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UT
SS
LEC
E
SCR
1
SCR
2
TM
CD-1
CD-2
Human LECAM1
1 kb
5'
UT
SS
LEC
E
1 2 3 4 5 6
SCR
TM
1 2
CD
3'
UT
Human LECAM2
5 kb
5'
UT
SS
LEC
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6
SCR
7 8 9
TM
1 2
CD
3'
UT
Human LECAM3

FIG. 2

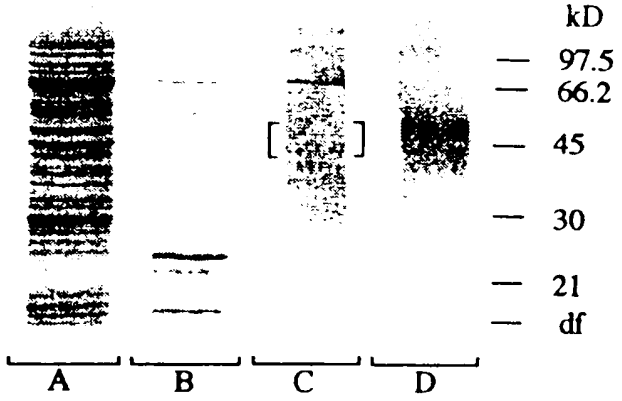
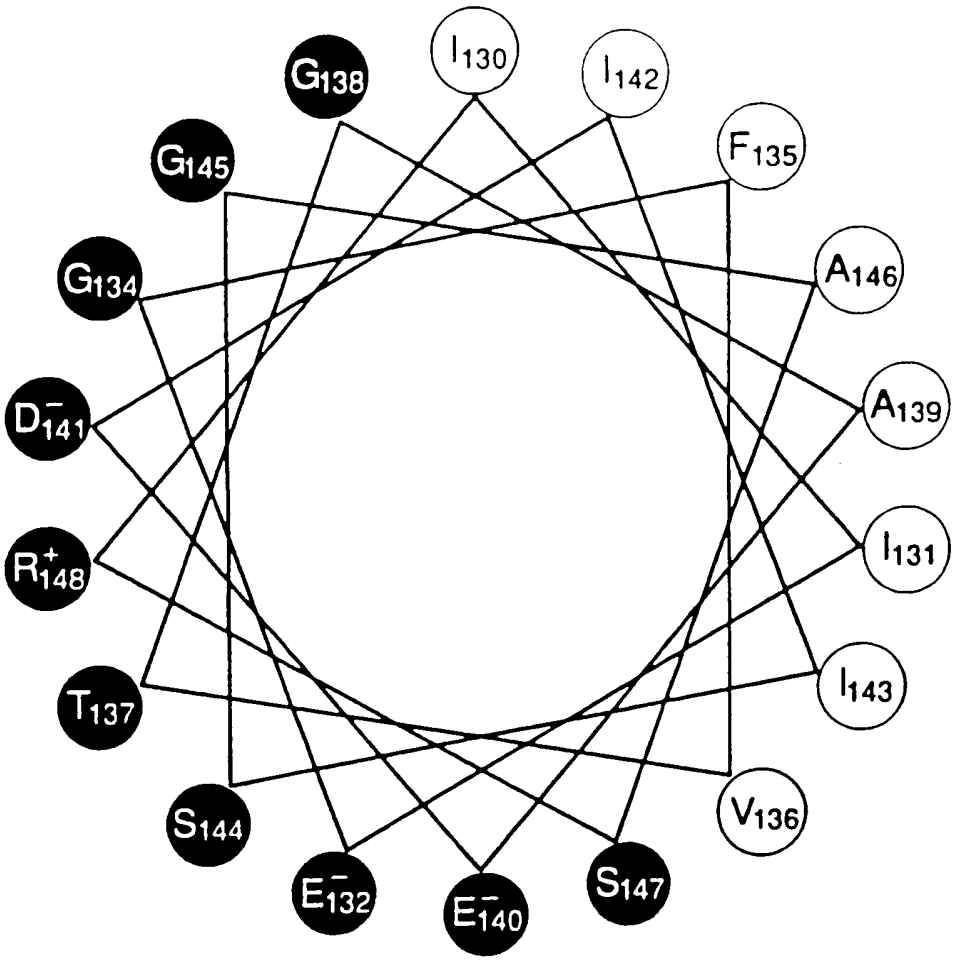


FIG. 3A

L	P	G	S	K	D	E	L	Q	M	K	T	Q	P	M	D	A	I	P	A	A	Q
				Q							R		Y								

FIG. 3B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I142
F135
A146
A139
I131
I143
V136
S147
E-140
E-132
S144
T137
R+148
D-141
G134
G145
G138

FIG. 3C

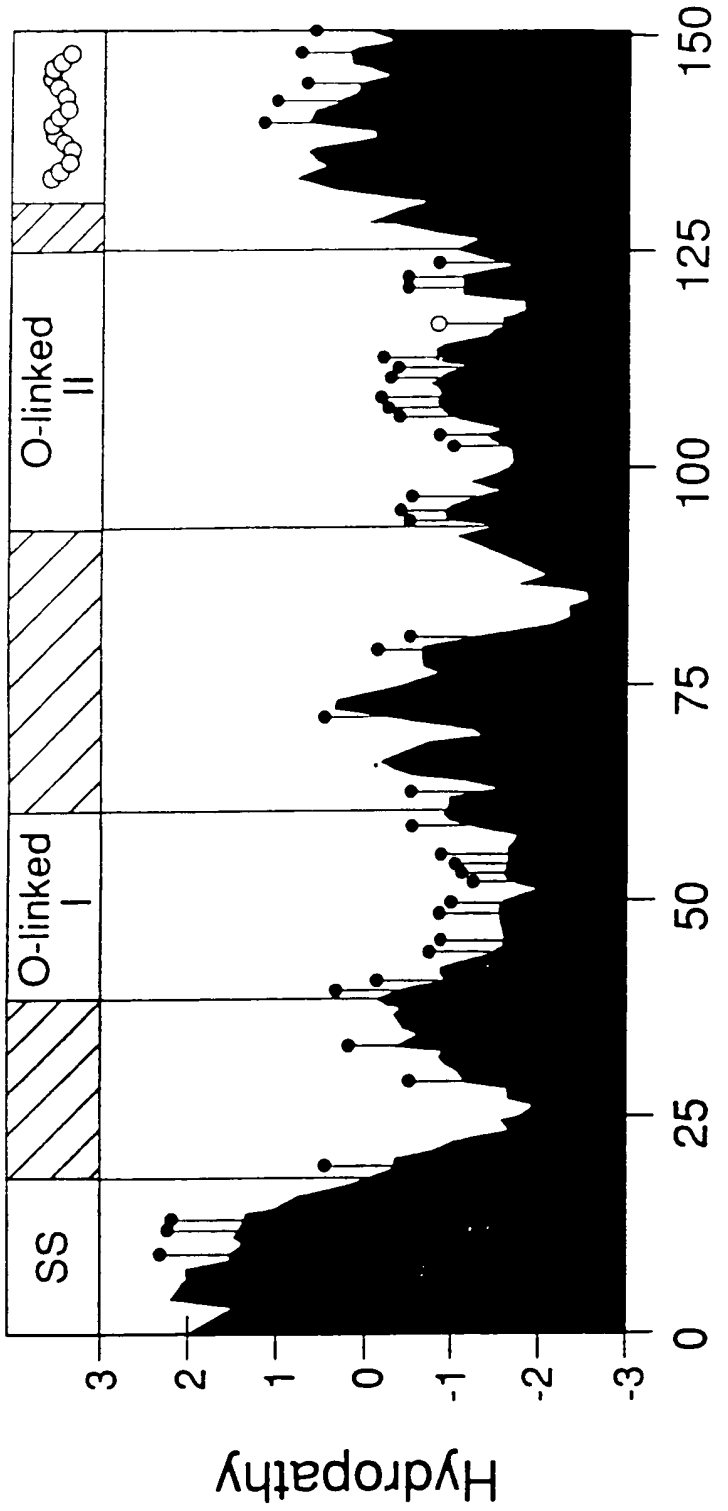
SS
O-linked I
O-linked II
3
2
1
0
-1
-2
-3
Hydropathy
0
25
50
75
100
125
150
Amino Acid

FIG. 3D

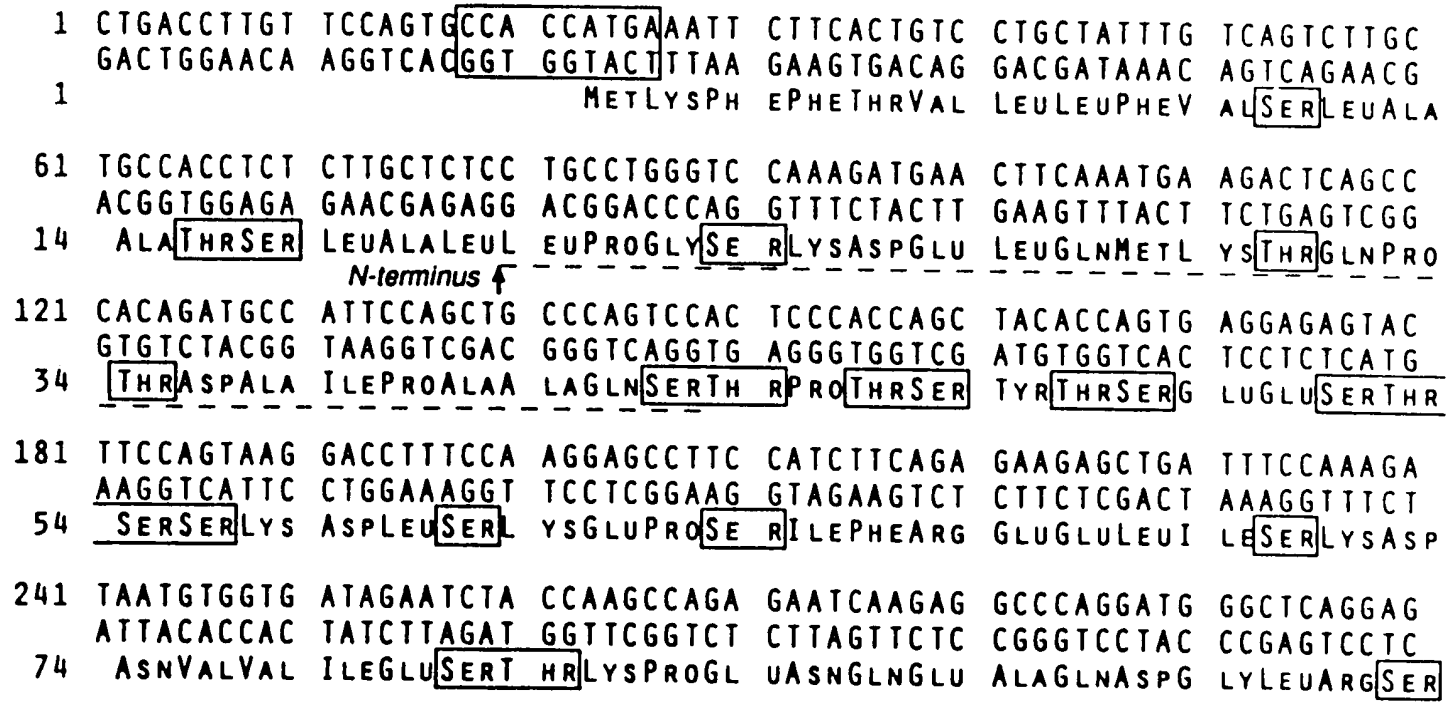

FIG. 4A

```
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    GCCCAGTAGA GTCGACCTTC TCTGGTGTTC TGGGTGGTGG AGTCGACGTT GGTGGAGTCT
 94   GLYSERSER GLNLEUGLUG LUTHRTHRAR GPROTHRTHR SERALAALAT HRTHRSERGLU

361 GGAAAATCTG ACCAAGTCAA GCCAGACAGT GGAGGAAGAA CTGGGTAAAA TAATTGAAGG
    CCTTTTAGAC TGGTTCAGTT CGGTCTGTCA CCTCCTTCTT GACCCATTTT ATTAACTTCC
114   GLUASNLEU THRLYSSERS ERGLNTHRVA LGLUGLUGLU LEUGLYLYSI LEILEGLUGLY

421 ATTTGTAACT GGTGCAGAAG ACATAATCTC TGGTGCCAGT CGTATCACGA AGTCATGAAG
    TAAACATTGA CCACGTCTTC TGTATTAGAG ACCACGGTCA GCATAGTGCT TCAGTACTTC
134   PHEVALTHR GLYALAGLUA SPILEILESE RGLYALASER ARGILETHRL YSSER

481 ACAAAAACAC CTAACCACTA AGTCCCATGC TAGGTGGTGC CTTCATCAGC CACATTCTGC
    TGTTTTTGTG GATTGGTGAT TCAGGGTACG ATCCACCACG GAAGTAGTCG GTGTAAGACG

541 TCATCTGACC ACCACCTCTC AGTCTGCCCT TTGATGTCTT ACATTAAAGT ATTGCAACCT
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601 AAAAAAAAA
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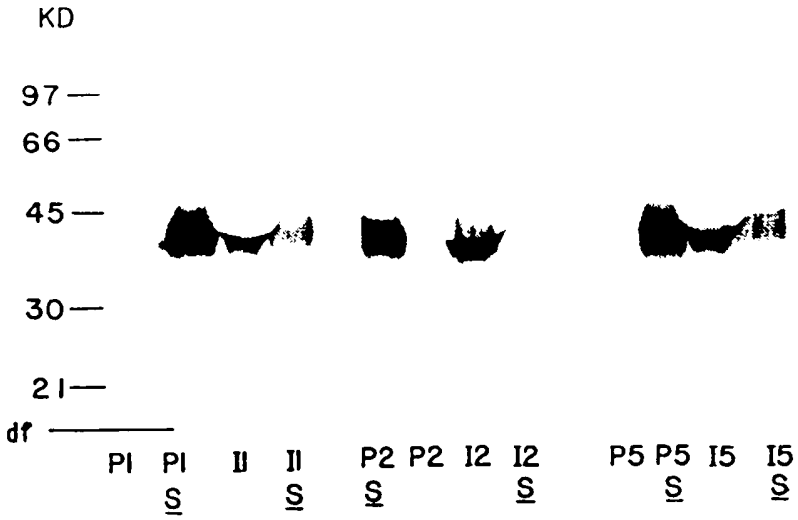
FIG. 4B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97
66
45
30
21
df
P1 P1 S I1 I1 S P2 S P2 I2 I2 S P5 P5 S I5 I5 S

FIG. 5A

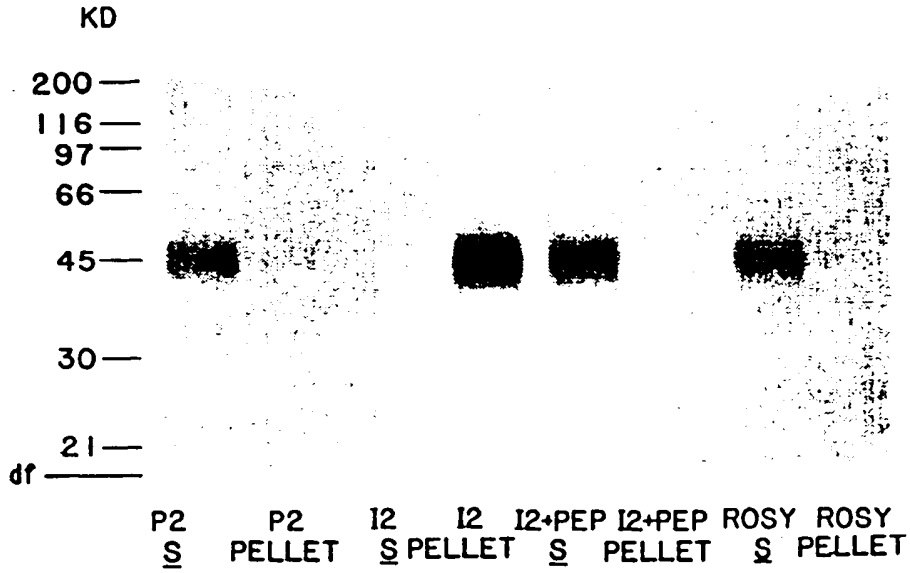
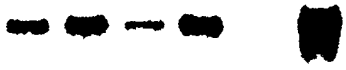

KD
200
116
97
66
45
30
21
df
P2 S
P2 PELLET
I2 S
I2 PELLET
I2+PEP S
I2+PEP PELLET
ROSY S
ROSY PELLET

FIG. 5B

FIG. 6A

FIG. 6B



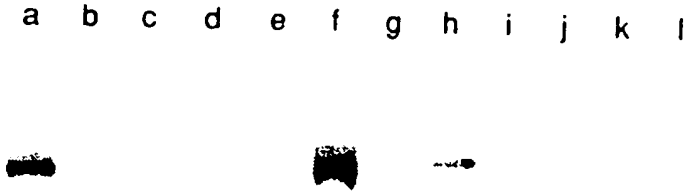


FIG. 6C

FIG. 6D

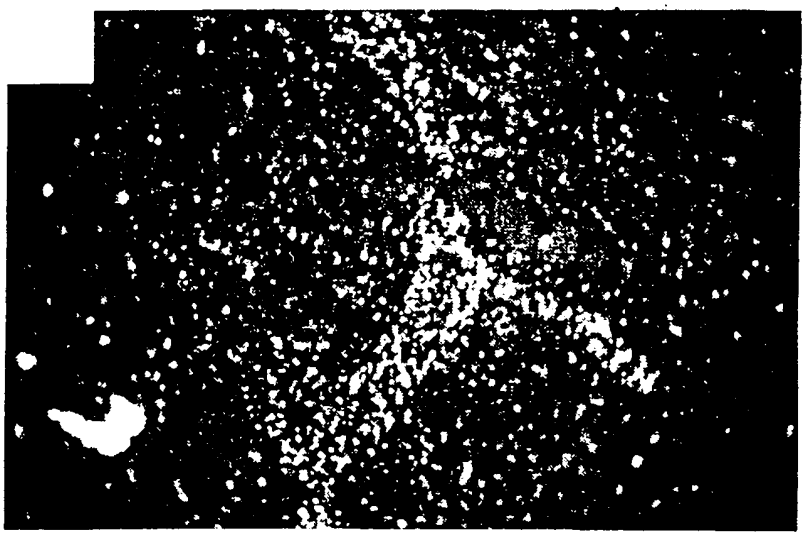


FIG. 7A

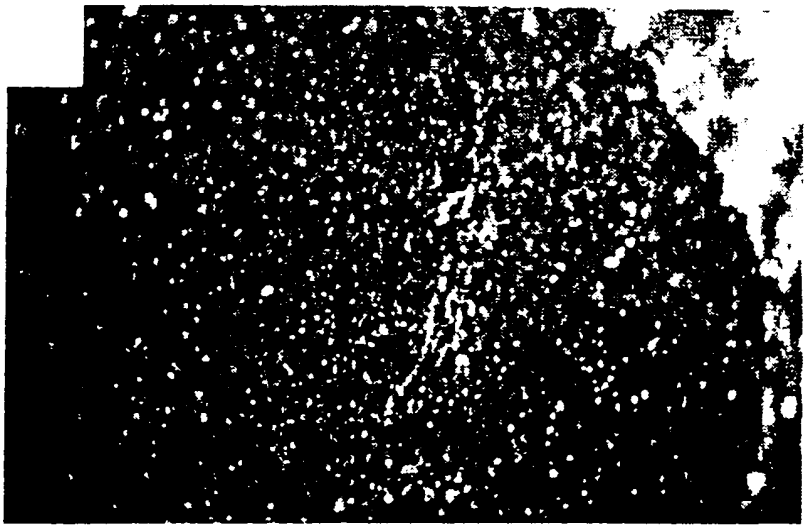


FIG. 7B

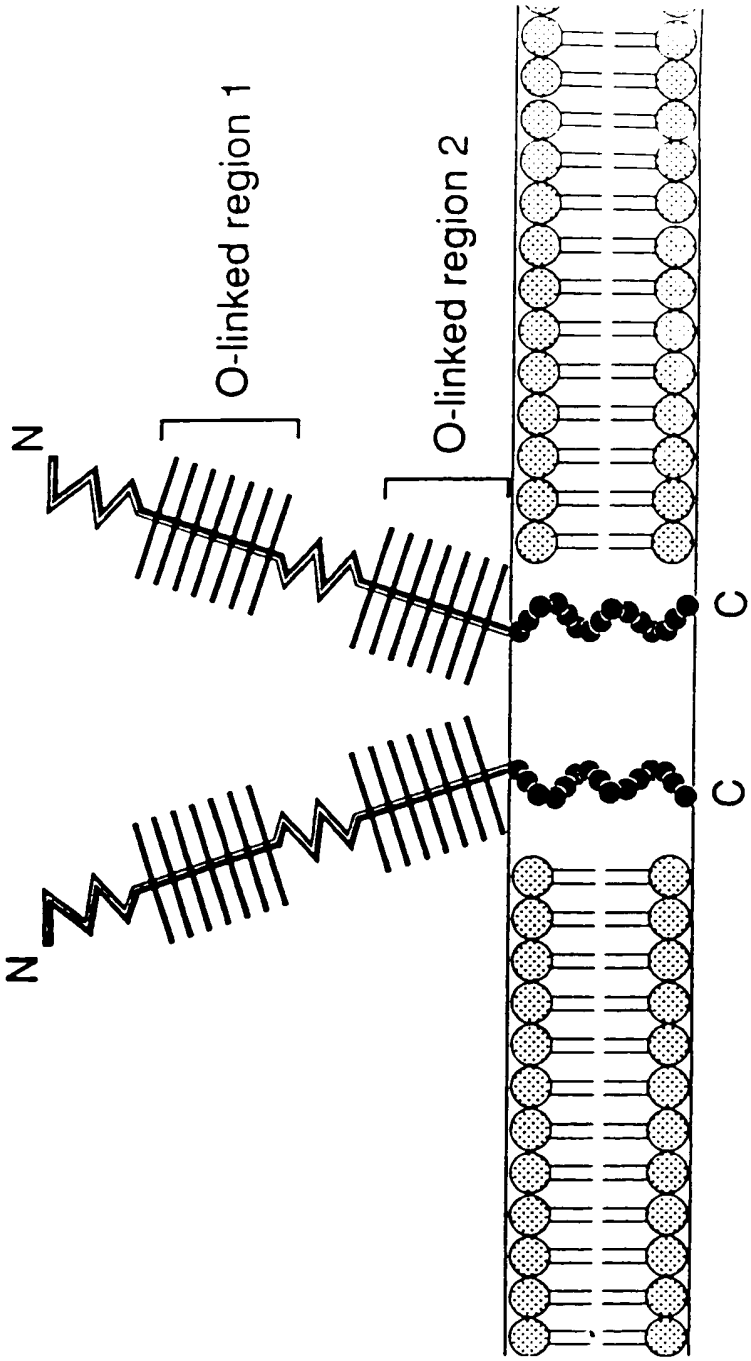


FIG. 8

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/03755

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/12; C07K13/00; C12P21/02; C12N15/62
C12N15/13; A61K37/02; C12P21/08; C12N5/10

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; C12N ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	WO,A,9 013 300 (BIOGEN, INC. USA) 15 November 1990 See pages 1-36; claims 1-10, 13-84, 116-118	1,2,5,8, 9,11-15, 18-20, 28,29, 31-34, 36-38
Y	NATURE. vol. 349, 28 February 1991, LONDON GB pages 796 - 799; Picker, L.J. et al.: 'ELAM-1 is an adhesion molecule for skin-homing T cells.' See the Abstract	1,2,5,8, 9,11-15, 18-20, 28,29, 31-34, 36-38

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17 SEPTEMBER 1992	
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	NAUCHE S.A.

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9203755 SA 60556

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 17/09/92

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9013300	15-11-90	AU-A-	6049290	29-11-90
		CA-A-	2031518	29-10-90
		EP-A-	0458911	04-12-91
		JP-T-	4502859	28-05-92

Contents:
The Journal of Cell Biology

Volume 118, Number 2, July 1992

Contents continued on reverse of this cover

Cover picture: P60c-src associated with endosomes is concentrated at the spindle poles during mitosis. A telophase rat-1 fibroblast over-expressing chicken c-src was processed for indirect immunofluorescence to visualize p60^{c-src} and data was collected by an optical sectioning microscope. Staining intensity is displayed as a pseudo-color gradient and demonstrates a concentration of p60^{c-src} at the spindle poles of dividing fibroblasts (*orange/yellow* representing the most intense staining). See related article in this issue by Kaplan et al., 321-333.